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(54) Title: OTITIS MEDIA VACCINE

(57) Abstract

It has been discovered that a vaccine comprised of fimbrin, a filamentous protein derived from the bacterial surface appendages of non-typable *Haemophilus influenzae* is useful in studying, preventing or reducing the severity of, otitis media. The gene sequence of the DNA coding for fimbrin and the amino acid sequence of fimbrin have also been determined. Vectors containing DNA coding for fimbrin have also been developed, and transformants have been prepared which contain such vectors and which express such DNA and provide a source of pure fimbrin.

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THE SPECIFICATION

OTITIS MEDIA VACCINE

This invention was made with government support in part under Grant No. DC00090 awarded by the National Institute of Health. The government has certain rights in this invention.

Background of the Invention

Otitis media is an infection of the middle ear that occurs primarily in children. Left untreated, the disease can result in hearing loss, and developmental delays. It is estimated that otitis media accounted for 31 million of the 130 million office visits for respiratory diseases in the period from 1987-87. Recent data indicate that suppurative and unspecified otitis media rank first in the list of the 30 most common diagnoses requiring a physician's office visit for patients up to age 24. Over one billion dollars per year is spent on treatment of this disease and the related loss of income for working parents is estimated to be between $300 and $600 million. Approximately 83% of all children by three years of age will have had at least one episode of acute otitis media. Non-typable strains of *Haemophilus influenzae* account for 25-30% of all cases of otitis media, 53% of recurrent otitis media, and are the primary pathogens isolated from 62% of cases of chronic otitis media with effusion. Although non-typable *Haemophilus influenzae* (NTHi) are

primary pathogens in otitis media, neither the pathogenic mechanisms nor the host immunological response has been fully defined for this disease.

It would be desirable to have a vaccine to confer immunity to non-typable *Haemophilus influenzae* or to reduce the severity of otitis media caused by *Haemophilus influenzae*.

Summary of the Invention

It has been discovered that a vaccine comprised of fimbrin, a filamentous protein derived from the bacterial surface appendages of non-typable *Haemophilus influenzae* is useful in studying, preventing or reducing the severity of, otitis media. The gene sequence of the DNA coding for fimbrin and the amino acid sequence of fimbrin have also been determined. Vectors containing DNA coding for fimbrin have also been developed, and transformants have been prepared which contain such vectors and which express such DNA and provide a source of pure fimbrin.

Brief Description of the Figures

Figure 1 is an A) Coomassie brilliant blue stained sodium dodecylsulfate-polyacrylamide gel electro phoretigram (SDS-PAGE) of: (a) molecular weight standards; (b) total outer membrane protein preparation from NTHi strain #1128 and (c) isolated fimbrin protein from strain #1128.

Figure 2 is a collection of light micrographs of H&E stained tissue sections of tympanic membrane from immunized chinchillas which received the homologous NTHi strain #1128. Chinchillas were immunized with: (A) control preparation; (B) total outer membrane protein #1128; (C) isolated fimbrin protein #1128; (D) isolated major outer membrane protein #1128. Micrograph (E) shows normal chinchilla tympanic membrane. All micrographs ar at a magnification of 210 x. The following designations are used to identify the following tissues: Ep - epidermal

lay r; CT - conn ctive tissue of fibrous lay r; MEM - middle ear mucosa; MEC - middle ear cavity; and RBCs - erythrocytes. Tympanic membrane (TM) of control chinchilla (A) demonstrates thickened and edematous CT layer. Note minimal thickening of tympanic membrane relative to normal (E) in B and C. Chinchillas immunized with the isolated major outer membrane protein of strain #1128 (D) demonstrate marked thickening of the tympanic membrane with bleeding evidenced by the presence of red blood cells connective tissue in the (RBCs) and edema in the fibrous layer (CT).

Figure 3 is a collection of light micrographs of H&E stained tissue sections of middle ear mucosa from immunized chinchillas which received the homologous NTHi strain #1128. Chinchillas were immunized with (A) control preparation; (B) total OMP #1128; (C) isolated fimbrin protein from NTHi 1128; (D) major outer member protein isolated from strain #1128. Micrograph (E) is that of normal chinchilla middle ear mucosa. All micrographs are at a magnification of 210 x. EX - exudate; MEC - middle ear cavity; MEM - middle ear mucosa; NB - new bone (osteoneogenesis); RBCs - erythrocytes; CT - connective tissue.

Figure 4A is a transmission electron micrograph of epon-embedded and thin sectioned NTHi strain #1128 showing thin, filamentous peritrichously arranged fimbriae.

Figure 4 (B) is a transmission electron micrograph of unfixed, unstained NTHi strain #1128 which has been indirectly immunolabeled with chinchilla anti-fimbrin protein antisera and gold-conjugated protein A and shadow cast. Fimbriae appear as white "rivulets" labeled with black gold spheres.

Figure 5 is the nucleotide sequence of NTHi fimbrin gene. The deduced amino acid sequence is shown below the DNA sequence. Capital letters correspond to th open reading frame. Amino acid sequences of the amino terminus

and an internal CNBr fragment determined by sequencing of the fimbrin protein are single underlined. The ribosome binding site is indicated by doubl und rlined. A stem-loop structure located downstream of the fimbrin gene is in boldface and underlined.

Figure 6 is a southern hybridization blot/analysis. Genomic DNA from NTHi #1128 parent strain were run in panel A, lanes 1, 2, 3, 4 and panel B, lane 1 and the DNA from the mutant strain were run in panel A, lanes 5, 6, 7, 8 and panel B, lanes 2, 3, 4, 5. DNA that was digested to completion with EcoRI was run in panel A, lanes 1 and 5 and panel B, lanes 1 and 2; EcoRI-HindlII (Panel A - lanes 2, 6 and Panel B - lane 3), EcoRI-PstI (Panel A - lanes 3, 7 and Panel B - lane 4) and TaqI (Panel A - lanes 4, 8 and Panel B - lane 5), electrophoresed on a 1% agarose gel, transferred to nitrocellulose membrane and probed with ^{32}P-labeled fimbrin gene (Panel A) and ^{32}P-labeled chloramphenicol acetyl-transferase gene (Panel B).

Figure 7 Western blot with chinchilla polyclonal antiserum directed against the upper band of the isolated fimbrin protein of the parental NTHi strain #1128 versus: (b) NTHi strain #1128 isolated fimbrin protein (upper band); (c) NTHi strain #1128 total outer membrane protein; (d) mutant strain total outer membrane protein; (e) mutant strain isolated lower band. Lane (a) contains prestained molecular weight standards.

Figure 8 shows a western blot analysis of: normal chinchilla serum pool in A & C serum obtained post-immunization with which isolated fimbrin protein from strain #1128 in B & D versus total outer membrane protein preparations from *Haemophilus influenzae* clinical isolates (non-typable and type b): (a) 86-042; (b) 86-043; (c) 1667 MEE; (d) 1128; (e) 1885 MEE; (f) 169 p+; (g) 90-100 L; (h) 90-100 R; (i) 90-111 L; (j) 90-112 R; (k) 90-114 NP; (l) 90-114 L; (m) Mr 13 p-; (n) Mr 13 p+; (o) Eagan p+; (p) Eagan p-.

Figure 9 is a w stern blot analysis of cell lysates prepared from *E. coli* BL21 (DE3)/pLys S transformed with pET3a (lane 1) and pNHF before (lane 2) and after (lane 3) induction with 0.5 mM IPTG. The blot was probed with polyclonal chinchilla serum directed against the isolated fimbrin protein from NTHi strain #1128 diluted 1:250.

Figure 10 shows the expression of fimbrin protein in recombinant (lane 1) and wild type (lane 2) baculovirus-infected cells. The infected cell extracts were analyzed by SDS-PAGE and western blotting with polyclonal chinchilla serum directed against the isolated fimbrin protein from NTHi strain #1128 (1:250 dilution) as the primary antibody.

Detailed Description of the Invention

It has been found that fimbriae, which are surface appendages, are produced by 100% of the bacteria recovered from the middle ears and nasopharynges of children with chronic otitis media. Fimbriae appear, via transmission electron microscopy, to be involved in the initial docking or adherence of the bacterial cell to mucosal epithelium.

It has also been discovered that vaccinating animals with fimbrin, a protein that comprises fimbriae, induces an immune response to the fimbrin protein,and protects the vaccinated animal from severe otitis media upon subsequent exposure to NTHi.

Immunogold localization of fimbriae.

Unfixed, unstained, immunogold-labeled whole bacteria were subjected to low angle platinum-palladium shadow casting to impart a sense of height to the micrograph in an attempt to more clearly resolve labeling of the low-profile NTHi fimbriae with both a battery of polyclonal and singular monoclonal antibody, designated as MAb 4A5u, directed against the isolated fimbrin protein. As shown in Figure 4, NTHi strain #1128, American Type Culture Collection,(ATCC) Number _____(number not yet assigned)

was labelled with a pool of chinchilla sera collected from a cohort immunized with the isolated fimbrin pr tein. Such lab lling indicated that th immunological response in chinchillas immunized with isolated fimbrin protein was directed against the fimbrae described on 100% of otitis media isolates examined.

Passive Immunization.

The protection conferred by an animal's immune response directed against the fimbrin subunit protein was determined in a chinchilla model of experimental otitis media. Chinchillas were passively immunized with 5 ml/kg hyperimmune chinchilla or rabbit serum directed against fimbrin protein isolated from NTHi strain 1128. Control chinchillas received normal rabbit serum or normal chinchilla serum. Next the chinchilla received transbullar challenge with the homologous NTHi, that is, 2.5 to 3.5 cfu/ear of NHTi strain #1128. The chinchillas were examined and rated. As shown in Table 1, the immunized chinchillas receiving immune rabbit or chinchilla serum displayed reduced tympanic membrane pathology ($p \leq 0.05$ and 0.001 respectively). As shown in Table 2, the presence of middle ear fluids in chinchillas receiving chinchilla anti-fimbrin protein serum were reduced when compared to controls.

Table 1

Intensity of Otoscopically Determined Tympanic Membrane Pathology in Passively Immunized Chinchillas Post-Intrabullar Challenge with NTHi

Group	Antisera 5 ml/kg		Days Post-Intrabullar Challenge (a)																				
			1	2	3*	4	5	6	7*	8	9	10*	11	12	13	14*	15	16	17*	18	19	20	21
A	Rabbit anti-NTHi Fimbrial Subunits 1:10	Left	1+	1+	1+	-	-	2+	2+	2+	2+	2+	-	-	2+	2+	1+	1+	1+	-	-	1+	1+
		Right	0	0	0	-	-	0	1+	0	0	0	-	-	0	0	0	0	0	-	-	0	0
B	Rabbit anti-Fimbrial Subunits 1:100	Left	1+	-	2+	1+	2+	2+	1+	-	-	3+	-	-	3+	3+	-	-	3+	-	-	1+	1+
		Right	0	-	0	0	0	+	0	-	-	1+	-	-	1+	0	-	-	0	-	-	1+	1+
C	Normal Rabbit Serum Undiluted	Left	2+	3+	2+	3+	3+	2+	2+	2+	3+	2+	1+	-	1+	2+	3+	3+	2+	1+	1+	2+	2+
		Right	0	0	0	1+	1+	-	1+	0	0	0	1+	-	0	0	1+	0	0	0	0	0	0
D	Normal Rabbit Serum 1:100	Left	2+	2+	2+	-	-	2+	2+	2+	3+	1+	-	-	1+	1+	+	0	0	-	-	1+	1+
		Right	0	1+	1+	-	-	1+	2+	1+	1+	1+	-	-	1+	1+	0	0	0	-	-	0	0
E	Chinchillas anti-NTHi Fimbrial Subunits Undiluted	Left	1+	1+	1+	1+	0	0	0	-	-	0	0	1+	0	0	-	-	0	0	0	0	0
		Right	0	1+	0	0	0	0	0	-	-	0	0	0	0	0	-	-	0	0	0	0	0

Group	Antisera 5 ml/kg		Days Post-Intrabullar Challenge (a)																				
			1	2	3*	4	5	6	7*	8	9	10*	11	12	13	14*	15	16	17*	18	19	20	21
F	Chinchilla anti-Fimbrial Subunits 1:100	Left	1+	1+	1+	1+	1+	1+	1+	-	-	0	0	0	0	0	-	-	0	0	0	0	0
		Right	0	1+	1+	0	0	0	0	-	-	0	0	0	0	0	-	-	0	0	0	0	0
G	Normal Chinchilla Serum Undiluted	Left	2+	2+	2+	2+	1+	1+	1+	-	-	1+	1+	1+	1+	1+	-	-	0	0	0	0	0
		Right	1+	0	1+	1+	1+	1+	0	-	-	0	0	0	0	0	-	-	0	0	0	0	0

* Epitympanic tap performed post-otoscopy on these days.
(a) Degree of pathology was graded on a 0 to 4+ scale, with 0 = normal drum appearance and 4+ = severe pathology, perforated drum with discharge. Number shown is average for each group.

Group A, B, E-G had 5 animals each.
Group C & D had 4 animals each.

Table 2

Presence of Middle Ear Fluids (MEF) in Chinchillas Receiving NCS or CαF Serum

Days Post-Inoculation	Presence of MEF in Challenged Ears # pos. ears/total		
	NCS UD	CαF UD	CαF 1:100
1	5/5	4/5	3/5
2	5/5	3/5	1/5
3	5/5	2/5	0/5
4	5/5	0/5	1/5
5	5/5	0/5	0/5
6	5/5	0/5	1/4(a)
7	5/5	0/5	1/4
10	5/5	0/5	0/4
11	5/5	0/5	0/4
12	1/5	0/5	1/4
13	3/5	0/5	0/4
14	1/5	0/5	0/4
17	0/5	0/5	0/4
18	0/5	0/5	0/4
19	0/5	0/5	0/4
20	0/5	0/5	0/4
21	0/5	0/5	0/4

(a) One animal died of undeterminate cause

NCS: normal chinchilla serum
CαF: chinchilla anti-fimbrial serum
UD: undiluted

To prepare vaccines for active immunization to NTHi, several NTHi prot ins wer isolated: the fimbrin protein from NTHi strain 1128; the fimbrin protein fr m NTHi strain 1885 ATCC Number _____(number not yet assigned) and the total outer membrane protein from NTHi strain 1128. While NTHi strains 1128 and 1885 have been described herein, other non-typable *Haemophilus influenzae* strains may be used including the publicly available strains publicly available from the ATCC number 43041.

Isolation of the fimbrin and the total outer membrane protein

The outer membranes proteins were isolated according to a modified procedure based on Carlone et al., "Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from Haemophilus species." (1986), J. Clin. Microbiol...24:330. NTHi strain 1885 and strain 1128 were each cultured as follows. The NHTi were grown for 18 hours in Brain Heart Infusion Broth containing: 2 mg. NAD/1; 2 mg. hemin/1 and incubated at 37 C. in a humidified atmosphere of 5% CO2 and 95% air. Then the NTHi were collected by centrifugation at 4000 x g for 20 minutes at 4°C, and decanted. The NTHi pellets were resuspended in 10mM HEPES buffer, pH 7.4, and sonicated for three 20 second pulses on ice using an Artek Sonic Dismembrator, Model 150 from Artek Systems Corp. at a setting of 60%. The sonicates were centrifuged at 9100 x g for 5 minutes at 4°C. The pellets were collected and the supernatant was centrifuged again to recover the crude outer membrane fraction. The pellets were combined and resuspended in 10 mM HEPES buffer and mixed in equal volumes with 2% sarcosyl (w/v) in the 10 mm HEPES buffer. The suspensions were incubated at room temperature for 60 minutes with occasional shaking. The suspension was then centrifuged at 5900 x g for 30 minut s at 4°C and th pellet was collected. Th p llets were gently surface-wash d with 200 ml double

distilled water without r suspending the pellets. The pellets were individually r suspended in 20 ml double distilled water to provide a outer membrane protein suspension. The outer membrane protein suspension was then aliqouted, frozen and maintained at 70°C. The total outer membrane protein isolated in the above described manner, from NHTi strain 1128 was then used as an immunogen for active immunization of animals.

To isolate the fimbrin protein, aliquots of the total outer membrane suspension were applied to large, 5-20% continuous gradient polyacrylamide gels known in the art as "slab" gels. The slab gels were run at 30 mA/gel for approximately 4 hours and rinsed in water. The slab gels were negatively stained with ISS Pro-Green staining system, available from Integrated Separation Systems according to the manufacturer's instructions for 10 minutes or overnight. The fimbrin band was identified via its migration relative to molecular mass standards run in adjacent lanes. The 25.5 kD band was excised from the gel using a razor blade, to obtain the fimbrin protein, although the 37.5 kD band may also be used, if the 37.5 kD fimbrin protein can be reassembled to its secondary structure. The 37.5 kD band contains the fully denatured form of the fimbrin protein. To obtain the fimbrin protein, the entire 25.5 kD bands were excised and cut into pieces approximately 1 cm. in length. The bands were destained according to the instructions provided by Integrated Separation Systems. Next, four to six gel pieces were placed in electroelution tubes and subjected to electroelution for 4 hours at 9 mA/tube. The electroeluted protein was collected in the reservoir tip of the electroelution tube from Bio-Rad Electro-Eluter and membrane caps w/12,000 MWCO. The electroluted proteins were dialyzed against distilled water for about 24 hours using 10,000 molecular weight cut ff dialysis membrane available from Spectrum Micro-ProDiCon Houstan Texas. The

above procedures were repeated, usually twice, until silver staining of lectrophoresed SDS-PAGE preparation indicated a lack of contamination with other uter membrane proteins. The fimbrin protein isolated in the above described manner, from NTHi strain 1128 and 1885 was also used as an immunogen for the active immunization of animals.

The outer membrane protein preparations were additionally observed via transmission electron microscopy of negatively stained preparations to confirm the reassembly of the isolated fimbrial protein into filaments upon dialysis.

Active Immunization

Five cohorts of 10 chinchillas each were actively immunized with either a saline control preparation or one of the following immunogens: a total outer membrane protein preparation from strain #1128; isolated fimbrin protein from NTHi strain #1128; isolated fimbrin protein from NTHi strain #1885; or an isolated major outer membrane protein approximately 40.5 kDa which constitutes the predominate outer membrane protein of strain #1128 but which is unrelated to the fimbrin subunit. The 40.5 kD major outer membrane is also known in the art as the "P2" protein. All immunogens were assessed for endotoxin content prior to their use as an immunogen via a chromogenic Amoebocyte Lysate assay which is commercially available from Whittaker Bioproducts under the designation QCL-1000. The chinchillas were subcutaneously injected with 100 μg immunogen in complete Freund's adjuvant. Then 30 days later they received 50 μg of the same immunogen in incomplete Freund's adjuvant. Following the second immunization, these five cohorts were divided into two groups each and challenged transbullarly with either strain #1128 or #1885. The chinchillas were assessed over a 4-we k period for: tympanic membrane pathology by otoscopic examination; semiquantitation of NTHi recovered via

epitympanic tap of th inf rior bulla; and light microscopic examination of fixed middle ear mucosal epithelium and tympanic membrane for histopathology.

As shown in Tables 3 and 4, the total outer membrane protein preparation and isolated fimbrin protein from strain #1128 were equally effective in significantly reducing tympanic membrane pathology ($p \leq 0.001$) for chinchillas challenged with the homologous fimbriated NTHi strain that is with NTHi strain 1128. Immunization with total outer membrane protein from strain 1128 also protected against the heterologous challenge with NTHi strain 1885 ($p \leq 0.001$) and was more likely to render middle ears effusion-free or culture-negative than immunization with the fimbrin protein. Immunization with fimbrin protein derived from strain 1885 was somewhat less protective against both homologous challenge ($p \leq 0.01$) and heterologous challenge ($p \leq 0.02$). Immunization with the major outer membrane protein, weighing approximately 40.5 kDa, did not protect against challenge with either strain 1128 or 1885. Indeed chinchillas receiving the approximately 40.5 kDa major outer membrane protein demonstrated significantly worse tympanic membrane pathology upon otoscopy ($p \leq 0.005$).

The chinchillas immunized with the control saline preparation demonstrated moderate histopathology of both tympanic membranes and middle ear mucosa. As shown in Figure 2, tympanic membranes were thickened with an edematous fibrous layer, whereas middle ear mucosa specimens demonstrated minimal thickening of the mucosa, osteoneogenesis and the presence of both red blood cells and inflammatory cells in the subepithelial space. A dense polymorphonuclear leukocytic exudate was present in the middle ear cavity.

Chinchillas immunized with either total outer membrane protein, or fimbrin protein isolated from NTHi strain 1128 demonstrated reduced tympanic membrane histopathology

compared to the control chinchillas. The administration of total outer membrane protein, rather than fimbrin protein, mor often resulted in effusion-fre ears or sterile effusions. As shown in Figure 3, the administration of total outer membrane protein resulted in an absence of a polymorphonuclear leukocytic comprised exudate overlying the middle ear cavity. Figure 3 reveals minimal thickening of mucosal layer in control chinchillas (A) relative to normal (E). There is a dense polymorphonuclear leukocytic exudate present in the middle ear cavity which is typically seen post-induction of otitis media with NTHi. Chinchillas immunized with total outer membrane protein (B) demonstrate significant thickening of the CT layer of the mucosa with bleeding into the subepithelial space as evidenced by the presence of red blood cells and some new bone formation. Chinchillas immunized with fimbrin protein (C) are similar to those immunized with total outer membrane protein (B) but with the addition of a predominantly polymorphonuclear leukocyte comprised exudate in the middle ear cavity. Chinchillas immunized with the isolated major outer membrane protein of strain 1128 (D) demonstrated similarly inflamed middle ear mucosa (as did all NTHi-challenged chinchillas) with the additional of extensive osteoneogenesis, a more predominant mononuclear character to the exudate and evidence of focal desquamation of the epithelial layer of the middle ear membrane, the severity of which was not seen in other cohorts.

Thus antibodies induced by vaccination with fimbrin or outer membrane protein and directed against fimbrin protein contribute to protection against NTHi-induced otitis media.

Since the fimbrin protein, whether isolated from NTHi or present as a component in a total outer membrane protein preparation, provides protection against otitis media by active and passive immunization, it is suitable for use immunization agent. In order to afford the broadest range of pr tection, a vaccinogen should elicit an immune

response that is both protective and broadly cross-reactive. Since ther is consid rable heterog neity among otitis media isolates of NTHi, total outer membrane proteins were isolated from the bacterial outer membranes of 15 randomly selected type b and non-typable clinical isolates of *Haemophilus influenzae*. To determine the extent of protection and cross reactivity of the vaccine, the bacterial outer membranes were solubilized in detergent and subjected to a Western blot of SDS-PAGE with polyclonal chinchilla antiserum directed against the isolated fimbrin protein from NTHi strain 1128. As shown in Figure 8, the Western blot showed that the polyclonal chinchilla antiserum recognized similarly migrating bands in all 15 of the bacterial outer membrane isolates indicating that the fimbrin protein in each of the 15 strains are serologically related. Therefore, the fimbrin proteins from the 15 different strains share common epitopes. Thus, fimbrin isolated from NTHi 1128 strain is a particularly suitable immunogen to protect against the different non-typable *H. influenzae* that cause otitis media.

Table 3
Active Immunization Trial
Average Tympanic Membrane Pathology (n = 5 ears)

Immunogen	Challenge NTHi Strain	Days Post-Intrabullar Challenge															
		1*	2	3*	4	7*	8	9	10*	11	14*	15	16	17*	18	28
Control	1128	2+	2+	2+	3+	2+	3+	3+	3+	3+	3+	2+	2+	2+	3+	2+
	1885	1+	1+	2+	1+	2+	2+	2+	2+	1+	2+	2+	1+	2+	1+	1+
NTHi #1128 total OMP	1128	1+	2+	2+	2+	2+	2+	2+	2+	2+	1+	1+	1+	1+	1+	1+
	1885	1+	2+	2+	1+	1+	1+	1+	1+	1+	1+	1+	1+	0	0	0
NTHi #1128 major OMP	1128	2+	3+	4+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
	1885	1+	2+	3+	2+	3+	3+	3+	3+	3+	3+	3+	2+	2+	2+	2+
NTHi #1128 fimbrial protein	1128	2+	2+	1+	2+	2+	1+	1+	1+	1+	1+	1+	2+	1+	1+	0
	1885	1+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	1+
NTHi #1885 fimbrial protein	1128	2+	3+	3+	3+	3+	3+	4+	3+	3+	3+	3+	3+	2+	2+	1+
	1885	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	0

Challenge dose: 2.5 - 3.5 E 3 c.f.u.
* Denotes day of epitympanic tap
OMP - outer membrane protein

Table 4
Active Immunization Trial
Statistical Comparison by Group

Immunogen	NTHi Challenge Strain	p-value	Status Relative to control
Control	1128	ND	
1128 total OMP		p≤0.001	+
1128 major OMP		p≤0.005	-
1128 fimb. prot.		p≤0.001	+
1885 fimb. prot.		p≤0.002	NS
Control	1885	ND	
1128 total OMP		p≤0.001	+
1128 major OMP		p≤0.001	-
1128 fimb. prot.		p≤0.13	NS
1885 fimb. prot.		p≤0.01	+

\+ indicates less tympanic membrane pathology relative to control
\- indicates greater tympanic membrane pathology relative to control
"p" indicates probability

Cloning and sequencing of the fimbrin gene.

Chromosomal DNA isolated from NTHi strain 1128 was sh ared by sonication and DNA fragments ranging from 2 to 5 kb were isolated using a 1% agrose gel. The fragments were attached to the Not-EcoRI linker-adapters from Stratogene Co and ligated with λgt11 arms from Stratogene Co. The ligated DNA was packaged *in vitro* into lambda particles by using Gigapack Plus from Stratagene according to the manufacturer's instructions, to provide a genomic library. To screen the genomic library by plaque hybridization, a 624 base pair polymerase chain reaction product probe was prepared as described below.

First the fimbrin protein from strain 1128 isolated as described above was digested with CNBr by suspending 500 ug fimbrin protein in 100 ul of 70% formic acid with 500 ug CNBr in 70 % formic acid and 5ug tryptophan. The digested protein was recovered and rinsed several times with distilled water.

The digested protein fragments were applied to a polyacrylamide gel then run at the same conditions as described above, then transferred to an Imobilon membrane from Millipore Co. All bands containing above about 2 picograms were excised, then the protein fragments were commercially sequenced by University of Southern California, at Riverside, using an applied Biosystems 475 A pulsed liquid protein sequencer and Applied Biosystems Computing Integrator. The two most predominant bands containing the protein fragments produced the N-terminus and an internal peptide which yielded the following two sequences of 20 and 15 amino acids respectively: APQENTFYAGVKAGQGSFHD and VSKTFSLNSDVTFAF. Based on these amino acid sequences, two nucleotide sequences were synthesized using Applied Biosystems Synthesizer and purified through oligonucleotide cartridges from Applied Biosystems. The two nucleotide sequ nc s wer : a 20-mer oligonucl otide with 128-fold degeneracy corresponding to

Gln3 through Ala9: 5' CA(AG)GA(AG)AA(CI)AC(AGTC) TT(CI)TA(CT)GC 3' and a 18-mer oligonucleotide with 512-fold d gen racy corresponding to the Phe 15 through Asp10: 5' AAA(AGTC)GC(AG)A(AGTC)GT(AGTC)AC(GA)TC 3. The 18-mer oligonucleotide was used as a sense primer and the 20-mer-oligonucleotide was used as an antisense primer to amplify the genomic DNA fragment encoding the N-terminal region of the fimbrin protein. The polymerase chain reaction product was obtained by preparing a mixture containing combining 100 ng genomic DNA, 50 pmol of the 20-mer oligonucleotide primer, 50 pmol of the 18-mer oligonucleotide primer, 10 nmol of each deoxynucleoside triphosphate, and 5 units Taq DNA polymerase from GibcO-BRL in a final volume of 100 μl. The genomic DNA in the mixture was denatured at 94°C for about 1 minute, then annealed at 50°C for about 2 minutes, and extended at 72°C for about 2 minutes. A last elongation step was done at 72°C for about 10 minutes, to provide a mixture containing the polymerase chain reaction amplified product. The polymerase chain reaction amplified product was run on an agrose gel, then purified from the agarose gel and labeled with ^{32}P using the random labeling kit from Baringer Manheim Co. to provide a radio labeled 624 base pair polymerase chain reaction product probe.

The genomic library was screened by using the 624 base pair polymerase chain reaction product as a hybridization probe according to Saybrook, Fritsch and Mantiatis (1989)"Molecular Cloning a Laboratory Manual" 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.. The 624 base pair polymerase chain reaction product hybridized with 3 phage plaques from the genomic library. The hybridization was carried out overnight at 42 C with standard solutions as disclosed in Saybrook, Fritsch and Mantiatis (1989) "Molecular Cloning a Laboratory Manual" 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., containing 50% formamide, then filtered. The

filters were washed for 30 minutes at 65 C in 0.1% XSSC and 11% SDS, then exposed to x-ray film. The positive plaques were identified from the radiograms and recovered fr m agar plugs. The three DNA fragments from the phage plaques were designated clones "λFD 1", "λFD 2", and "λFD 3". The phage DNA was isolated, digested with ECoRi, isolated by spin elution. The DNA fragments were then subcloned into plasmid pUC18 which is available from Sigma. The *Haemophilus influenzae* DNA fragments inserted into these phages created plasmids designated "FD1", "FD2" and "FD3". Sequencing of these plasmids revealed that they encoded different overlapping portions of the fimbrin gene sequence but none of them contained the full length gene. Plasmids FD1 and FD3, which contain an overlap of 237 base pairs were used to construct a plasmid carrying the complete coding sequence as well as 5' and 3' flanking regions of the fimbrin gene. The EcoRI-HindIII fragment of plasma FD1, containing the 5' upstream region and the first 450 base pairs of the fimbrin gene was isolated and inserted in the EcoRI-HindIII digested and dephosphorylated plasma FD3 to create a plasmi designate "FD".

The nucleotide sequence of the fimbrin gene was determined from the insert fragment in plasmid λFD. Both strands of this insert were sequenced by the Sander dideoxy-mediated chain terminated method, according to Saybrook, Fritsch and Mantiatis (1989)"Molecular Cloning a Laboratory Manual" 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., using the commercially available sequencer Sequence 2.0 from U.S. Biochemical Co.. The DNA sequence and the deduced amino acid sequence are shown in Figure 5. The entire fimbrin gene contained a 1077 base pair Open reading frame, beginning with an ATG codon at position 406 and ending with a TAA stop codon at position 1085. The Open reading frame is preceded by a putative ribosom -binding site AGGA similar to the cons nsus sequence for *E. coli* and beginning eleven base pairs

upstream of the initiation codon. One stem-loop structure consistent with a rho-independent transcription terminator is located downstream of the open reading frame. Preceding the coding sequence for the mature fimbrin protein was encoded a leader peptide of 21 amino acid residues with the characteristics of a typical signal sequence. The fimbrin gene is first translated as a precursor form consisting of 359 amino acids and later the signal sequence is processed to yield the mature fimbrin protein consisting of 338 amino acids. The calculated molecular mass is 36.4 kDa, which is almost identical to the molecular mass of the upper band in the SDS-PAGE, shown in lane 3 of Figure 1A. This band is believed to be the true fimbrin protein. The deduced amino acid sequence for the fimbrin gene agreed with the amino acid sequences of the N-terminus and an internal peptide derived from CNBr cleavage of the purified fimbrin protein, shown in Figure 5.

The open reading frame coding for fimbrin protein described herein can be used to express the recombinant protein in *E. coli* or other expression systems. Two examples are described below.

Example 1

Two oligonucleotides based on the first six codons and the last six codons of the coding sequence of the fimbrin gene served as primers in a polymerase chain reaction to amplify the coding sequence of the fimbrin gene employing genomic DNA from NTHi #1128 as a template. The synthesized polymerase chain reaction product was double digested for 1 hour at 37 C with BamHI and NdeI and subcloned in the corresponding cloning sites of the expression vector pET3a from England Biolabs according to Alan H. Rosenberg, et al., Gene, 1987, 56:125, and ligated overnight at 14 C, using T4 ligase, to yield plasmid pNHF. The ligated DNA was transformed into *E. coli* DH5α and the desired construction was verified by restriction analysis with

BamH1 and Nde1. The vector pET3a and plasmid pNHF were transform d into *E. coli* BL21(DE3)/pLysS. Expression of the fimbrin gene product under the control of the ϕ10 promoter was achieved by induction of T7 RNA polymerase synthesis by the addition of 0.5 mmole IPTG. The whole cell protein profile of BL21(DE3)/pLysS[pNHDF] was analyzed and compared to the profile of BL21(DE3)/pLysS[pET3a]. Western blot analysis shown in Figure 9 showed that *E. coli* expressed the recombinant protein.

Example 2

Fimbrin protein can also be expressed using baculovirus vector according to Luckow, V.A., Recombinant DNA Technology and Applications, eds., Prokop, A. Bajpai, R.K. and Ho, C.S. (McGraw, Inc., NY) 1991, 1097, in insect cells. A recombinant pBacPAK transfer vector was constructed by cloning the polymerase chain reaction-amplified coding sequence of the fimbrin gene into the BamHI site of pBacPAK1 vector from Clontech Laboratories, Inc. Palo Alto, Ca. following the manufacturer's instructions. After screening for the correct orientation of the insert using Hind III digestion, the recombinant gene was incorporated into the viral genome by cotransfecting insect cells, *Sporodoptera frugiperda*, with a mixture of wild type viral DNA and transfer vector DNA. Individual plaques were obtained and the recombinant viruses were tested for expression of fimbrin protein. Western blot analysis shown in Figure 10, indicates that the insect cells expressed *H. influenzae* fimbrin protein.

The fimbrin protein expressed may be used as a vaccine to prevent and/or reduce the severity, to study, and to treat otitis media in animals.

Insertional mutagenesis of the fimbrin gene.

As shown in Figure 6, the results of genomic Southern hybridization analysis of DNA from NTHi #1128 cleaved with a variety of restriction enzymes indicates that only a single copy of the fimbrin gene is present in strain #1128. The 952 base pair SfuI fragment from pBR325, containing the gene encoding chloramphenicol acetyl-transferase was blunt ended using 8 K gm phosphatase, from Epicenter Technologies, Madison Wisconsin, and ligated with T4 ligase to the Bst EII digested plasmid FD dephosphorylated and filled with Klenow enzyme in the presence of the four deoxynucleoside triphosphates. This plasmid was transformed into competent *E. coli* DH5α and the transformants were selected on LB agar containing 100 λlg/ml ampicillin and 25 λlg/ml chloramphenicol. One recombinant was designated "NFM". Restriction enzyme mapping of this NFM strain verified the position of the chloramphenicol cassette and verified that a single copy of the gene was inserted. The pNFM plasmid was purified, linearized with BamHI and transformed into NTHi #1128, made competent by the M-IV method according to Herriot et. al. "J. Bacteriology" (1970) vol. 101 pp. 517-524, which is incorporated herein by reference. Mutants were selected on supplemented brain heart infusion agar containing 2 λlg/ml chloramphenicol. Genomic DNA isolated from one of these mutants and from the parent, 1128 was digested with EcoRI, EcoRI HindIII, EcoRI PstI and Taq I and analyzed by Southern hybridization. EcoRI and Taq I cleave once inside the chloramphenicol gene and HindIII cuts once within the fimbrin gene downstream the point of insertion of the chloramphenicol cassette. The 952 base pair SfuI fragment from pBR325 encoding the chloramphenicol gene and the 1077 base pair EcoRI-BamHI fragment encoding the fimbrin gene, were used as ^{32}P-labeled hybridization probes. The autoradiograms are shown in Figur 6. The mutant strain was compared with the parent strain #1128 by western blot

analysis with the polyclonal antifimbrial chinchilla serum prepared against the upper band to detect immunoreactive proteins from whole cell extracts. This cross-reactive band was absent in the mutant, as shown in Figure 7. Coomassie staining indicated two fimbrin protein bands, one at about 37.5 kDa and another at about 25.5 kDa corresponding to the bands in Figure 1.

This lower band from the fimbrin gene-disrupted mutant strain did not cross react with the antibodies prepared against the 37.5 kD fimbrin protein. Variable degrees of cross reactivity with the 25.5 kD band were seen with the parent. These results suggest that the protein in the lower band can associate with the 37.5 kD fimbrin protein. To determine whether the lower band found in the mutant is involved in fimbriae formation, the lower band from the parent strain and the mutant strain, were examined electron microscopically with and without the addition of the 36 kDa protein. Only the parent strain showed fimbriae and, therefore, the lower band seen in the mutant is unrelated to fimbriae.

Effect of fimbrin gene disruption on fimbrae.

While negative staining and immunogold labeling revealed a fimbrae on the parent strain, no surface appendages were found on the mutant strain. The mutant strain was found to be 32-26% less adherent than the parent strain to eukaryotic target cells.

The pathogenicity of the parent strain and the mutant strain were compared. Ten chinchillas were inoculated with the NTHi; 5 chinchillas received the parent strain and 5 received the mutant strain. Dosage received was: 3.3 E 3 cells of the parent strain and 4.0 E 3 cells mutant strain. The NTHi was inoculated into the left superior bulla of the chinchilla, and sterile saline was inoculated into the right superior bullae as a control. Th results are shown in Table 5. While differenc s in tympanic

membran pathology ver time wer not remarkable, survival rates were notably different betwe n the two strains. Labyrinthine involvement, that is th effect on the inner ear, manifested by balance disorder was noted in all of the chinchillas receiving the parent strain. In comparison, 3 of the chinchillas receiving the mutant strain developed mild to moderate labyrinthine involvement.

Table 5
Semi-quantitative Assessment of Viable Bacteria in Epitympanic Tap Fluids Post-Transbullar Challenge with NTHi strain #1128 and Mutant #1

Animal #	Strain Received	CFU/ml	
		Choc. agar	BHI
1	Parent	$>10^8$	$>10^8$
2	Parent	$>10^8$	$>10^8$
3	Parent	2.1 E 7	6.7 E 6
4	Parent	$>10^8$	2.0 E 7
5	Parent	Dry	Dry
6	Mutant	Dry	Dry
7	Mutant	Dry	Dry
8	Mutant	2.9 E 6	4.8 E 4
9	Mutant	6.9 E 5	4.1 E 4
10	Mutant	1.4 E 5	2.5 E 4

* Tap performed 4 days post-inoculation of left middle ear of all chinchillas.

In an intranasal challenge study, 12 chinchillas were inoculated via passive inhalation of approximately 10^8 cfu of either the parent strain or mutant strain. Assessment of tympanic membrane pathology, shown in Table 7, indicated significantly reduced pathology in chinchillas inoculated with the mutant strain. Labyrinthine involvement was markedly reduced in chinchillas receiving the mutant strain. By day 13 there were only 3 chinchillas left alive in the parent cohort compared to 6 in the mutant cohort.

Thus, the ability of the mutant strain to gain access to, survive and multiply in the middle ear cleft was significantly hampered.

Table 6
Labyrinthine Involvement in Chinchillas Receiving A Transbuller or Intranasal Inoculation of NTHi strain #1128 or Mutant #1

Severity of Disease

Post transbuller Inoc.	None	Mild	Moder.	Severe	None	Mild	Moder.	Severe
Day 3	*	-	-	-	*	-	-	-
Day 4	⊗	-	⊙	⊙	*	-	-	-
Day 5	⊙	-	⊙	⊗	⊗	⊙	-	-
Day 6	⊙	-	⊙	⊗	⊗	-	⊙	-
Day 7	⊙	-	-	⊗	⊗	⊙	-	-
Day 8	⊙	-	⊙	⊙	⊗	⊙	-	-
Day 9	⊙	-	-	⊗	⊗	⊙	-	-
Day 10	⊙	-	-	⊗	⊗	⊙	⊙	-
Day 11	⊙	-	-	⊗	⊗	⊙	-	⊙
Day 12	⊙	-	-	⊗	⊗	-	⊙	⊙
Day 13	⊙	-	⊙	⊙	⊗	-	⊙	⊙

* All animals ⊙ One animal

⊗ >1 animal but less than all in cohort - No animals

Post transbuller Inoc.	None	Mild	Moder.	Severe	None	Mild	Moder.	Severe
Day 3	*	-	-	-	*	-	-	-
Day 4	⊗	-	⊙	⊙	*	-	-	-
Day 5	⊙	-	⊙	⊗	⊗	⊙	-	-
Day 6	⊗	-	⊙	⊙	⊗	-	⊙	-
Day 7	⊗	-	⊙	-	⊗	⊙	-	-
Day 8	⊗	-	-	⊗	⊗	⊙	-	-
Day 9	⊙	-	-	⊗	⊗	⊙	⊙	-
Day 10	⊙	-	-	⊗	⊗	⊙	⊙	-
Day 11	⊙	-	-	⊗	⊗	-	-	⊙
Day 12	⊙	-	-	⊗	⊗	-	-	⊙
Day 13	⊙	-	-	⊗	⊗	-	-	⊙
Day 17	*	-	-	-	*	-	-	-

* All animals ⊙ One animal

⊗ >1 animal but less than all in cohort - No animals

Table 7

Day	Bacterial Count in Chinchillas Receiving Parent Strain	Bacterial Count in Chinchillas Receiving Mutant Strain
3	No detectable bacteria all but one ear dry	No detectable bacteria all ears dry
7	3.4 E 8 (R-#2) 6.4 E 8 (L-#2) 1.3 E 7 (R-#5) 2.6 E 9 (L-#5) 3.2 E 9 (R-#1)	1.0 E 8 (R-#6) 7.4 E 5 (L-#9) 4.0 E 6 (R-#9)
12	8.2 E 6 (R-#2) 5.9 E 8 (L-#2) 1.1 E 9 (R-#S) 1.9 E 9 (L-#5)	6.6 E 5 (R-#6) 1.9 E 5 (R-#8) 1.3 E 7 (L-#8)

While the vaccine containing the fimbrin protein has been administered in a carrier such as Freund's adjuvant to chinchillas, other carriers, including pharmacologically acceptable carriers, are also suitable.

The fimbrin protein is also provided to the host animal by administering transformed microorganisms, which contain the fimbrin gene and express the fimbrin protein, to the host animal. Such microorganisms include mucosal pathogens such Salmonella, Mycobacterium, or Adenovirus, which preferably are attenuated. The fimbrin produced by the transformant generates a protective immune response in the host. The transformant is administered in a suitable carrier.

Adherence of fimbriated clinical NTHi isolate to human oropharyngeal cells was inhibited in a dose-dependent manner by fimbrin protein isolated from NTHi strain 1128 but was not inhibited by the 40.5 KDa NTHi outer membrane protein. Thus fimbrin protein whether isolated from NTHI such as strains 1128 or 1885, or produced by recombinant DNA techniqu s, ar also administered to pr vent or reduce adherence of NTHi to host cells thereby preventing or

reducing the severity of otitis media. The fimbrin protein is administered, before or after infection with NHTi, such as by an intranasal spray comprising the fimbrin protein and a carrier.

5

We Claim:

1. A vaccine against non-typable Haemophilus influenzae, to be administered in animals, comprising fimbrin protein.

2. The vaccine of claim 1 wherein the fimbrin protein is isolated from non-typable Haemophilus influenzae.

3. The vaccine of claim 1 wherein the fimbrin protein is isolated from non-typable Haemophilus influenzae strain 1885.

4. The vaccine of claim 1 wherein the fimbrin protein is isolated from non-typable Haemophilus influenzae strain 1128.

5. The vaccine of claim 1 wherein the fimbrin protein is produced from recombinant DNA.

6. The vaccine of claim 1 further comprising a transformed microbial host containing DNA sequence coding for the fimbrin protein wherein the fimbrin protein is expressed in the animal.

7. A method for vaccinating animals against otitis media comprising the step of administering a vaccine comprising a carrier and fimbrin protein.

8. A method for treating otitis media in an animal infected with Haemophilus influenzae comprising the step of administering fimbrin protein via mucosal membranes of the animal.

9. Fimbrin protein having an amino acid sequence as shown in Figure 5.

10. The fimbrin protein, isolated from non-typable Haemophilus influenza, wherein the fimbrin protein is characterized in that it migrates in polyacrylamide gels to a position equivalent to a molecular weight of about 25.5 KD or about 37.5 KD.

11. A DNA sequence coding for fimbrin protein.

12. A DNA sequence as shown in Figure 5 coding for fimbrin protein.

13. A vector containing the DNA sequence of claim 11 coding for fimbrin protein.

14. A vector containing the DNA sequence of claim 12 coding for fimbrin protein.

15. The vector of claim 13 wherein the vector is a plasmid.

16. The vector of claim 13 wherein the vector is plasmid pET3a.

17. The vector of claim 13 wherein the vector is baculovirus.

18. A microbial host transformed by the vector of claim 13 containing the DNA sequence coding for fimbrin protein.

19. The invention of claim 18, wherein the host is E. coli.

20. The invention of claim 19, wherein the host is Sporodoptera frugiperda.

21. The invention of claim 20 wherein the host is a mucosal pathogen.

22. A process for the preparation of fimbrin protein comprising culturing a transformed microbial host of claim 18 under conditions suitable for the expression of fimbrin and recovering fimbrin.

23. A process for the preparation of fimbrin protein comprising culturing a transformed microbial host of claim 19 under conditions suitable for the expression of fimbrin and recovering fimbrin.

24. A process for the preparation of fimbrin protein comprising culturing a transformed microbial host of claim 20 under conditions suitable for the expression of fimbrin and recovering fimbrin.

25. Fimbrin protein made by the process of claim 22.

26. Fimbrin protein made by the process of claim 23.

27. Fimbrin protein made by the process of claim 24.

28. A biologically pure culture of non-typable Haemophilus influenza strain 1128.

29. A biologically pure culture of non-typable Haemophilus influenza strain 1885.

Figure 1

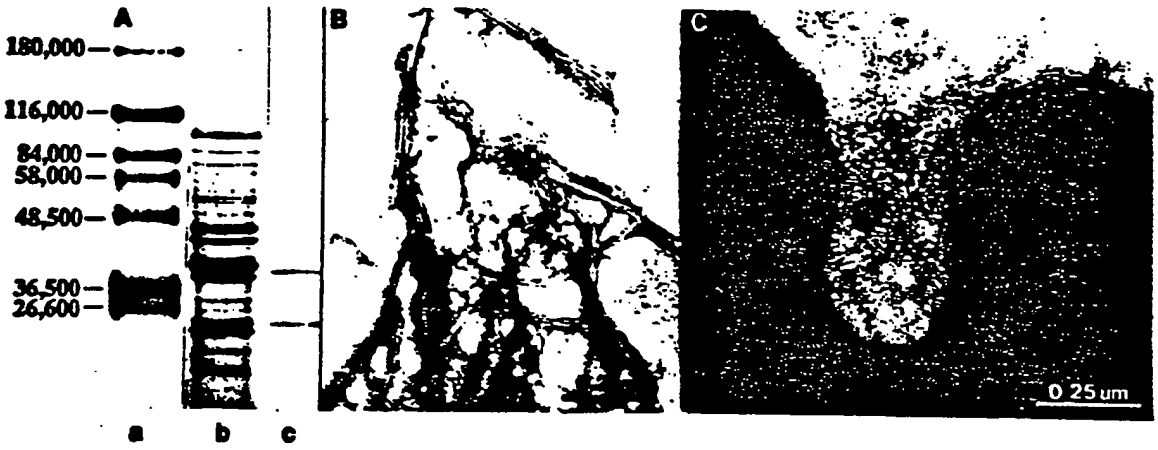

Figure 2

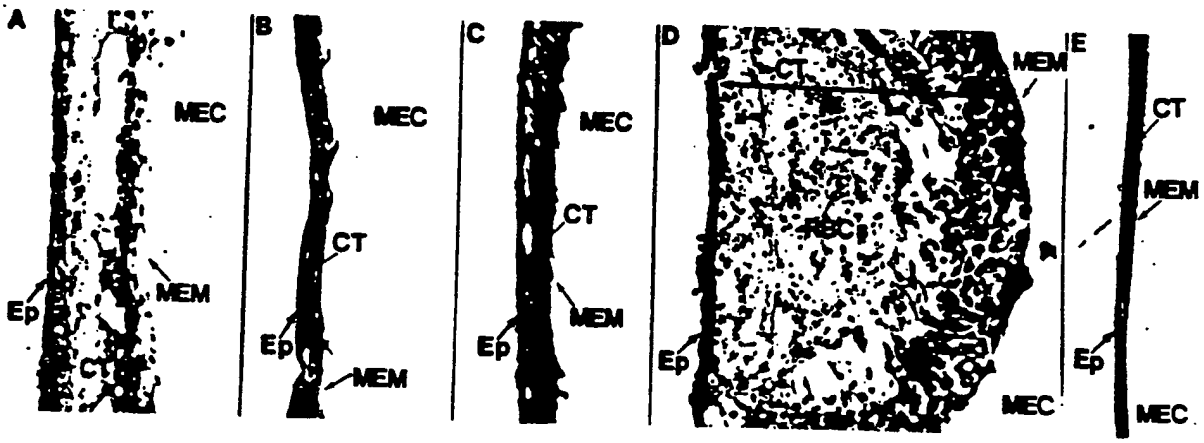

Figure 3

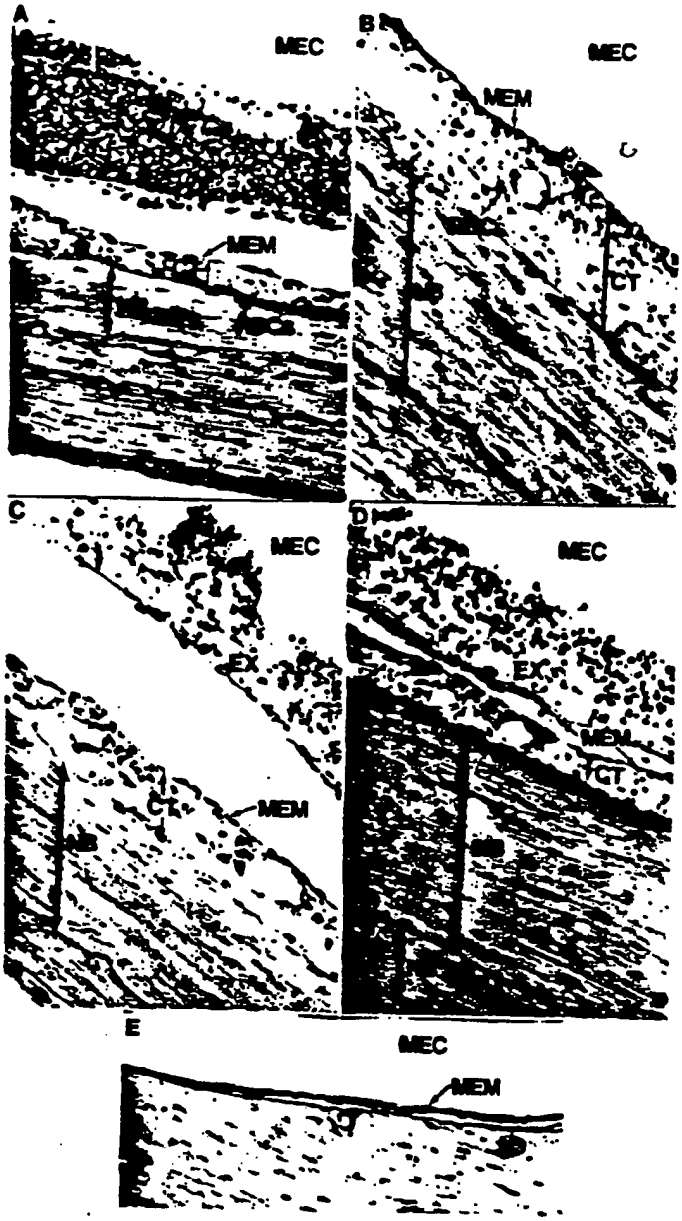

Figure 4

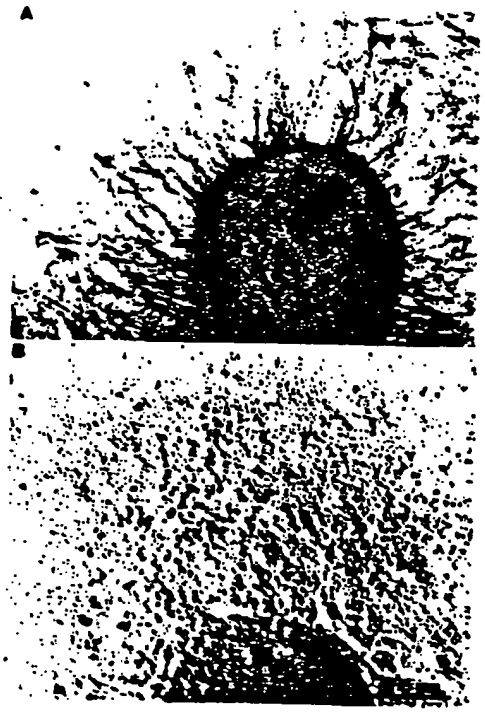

Figure 5

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   1 atgtcactgaggatgcgattagacctggccacatgctattaactcattaagctaaaatgg  60
  61 cagtctattgacctaatatcttaaggcgttaatgatgtcgaattagattttgagcattta 120
 121 agagtgtttatggagaaatgagtcaagaaagtgtgtgtttggatgttttcaataacaaaa 180
 181 attcaaaagatatgatcttttcaatttttataggataataagcgcacttttgaacgttcct 240
 241 ttggggtaaacataaacaaaggaattgaatttgtcaaaaggtagcaatgaggcaaattca 300
 301 aaccctcgttaagtgaactgtttagaagataactttgattaaaagttcggtctaaacggg 360
 361 aataatttttttattactattcgatgactaaatagaggacatcaaa ATG AAA AAA    414
   1                                                M   K   K      3
 415 ACT GCA ATC GCA TTA GTA GTT GCT GGC TTA GCA GCA GCT TCA GTA  459
   4 T   A   I   A   L   V   V   A   G   L   A   A   A   S   V   18

 460 GCT CAA GCA GCT CCA CAA GAA AAT ACT TTC TAC GCT GGC GTT AAA  504
  19 A   Q   A   A   P   Q   E   N   T   F   Y   A   G   V   K   33

 505 GCT GGT CAA GGA TCT TTC CAT GAT GGT ATT AAC AAT AAT GGC GCA  549
  34 A   G   Q   G   S   F   H   D   G   I   N   N   N   G   A   48

 550 ATT AAA AAG GGA TTA TCA TCT AGT AAT TAT GGT TAC AGA CGC AAT  594
  49 I   K   K   G   L   S   S   S   N   Y   G   Y   R   R   N   63

 595 ACT TTC ACT TAT GGT GTA TTT GGT GGT TAC CAA ATT TTA AAT CAA  639
  64 T   F   T   Y   G   V   F   G   G   Y   Q   I   L   N   Q   78

 640 GAT AAT TTT GGT TTA GCT GCT GAA TTA GGT TAC GAC GAT TTC GGT  684
  79 D   N   F   G   L   A   A   E   L   G   Y   D   D   F   G   93

 685 CGT GCA AAA CTT CGT GAA GCG GGA AAA CCT AAA GCT AAA CAT ACT  729
  94 R   A   K   L   R   E   A   G   K   P   K   A   K   H   T   108

 730 AAC CAC GGT GCG TAC TTA AGC TTA AAA GGC AGC TAT GAA GTG TTA  774
 109 N   H   G   A   Y   L   S   L   K   G   S   Y   E   V   L   123

 775 GAC GGT TTA GAT GTT TAT GGC AAA GCA GGT GTT GCT TTA GTA CGT  819
 124 D   G   L   D   V   Y   G   K   A   G   V   A   L   V   R   138

 820 TCT GAT TAT AAA TTT TAT GAA GAT GCA AAC GGT ACT CGT GAC CAC  864
 139 S   D   Y   K   F   Y   E   D   A   N   G   T   R   D   H   153

 865 AAG AAA GGT CGT CAC ACA GCA CGT GCC TCT GGT TTA TTT GCA GTA  909
 154 K   K   G   R   H   T   A   R   A   S   G   L   F   A   V   168

 910 GGT GCA GAA TAC GCA GTA TTA CCA GAA TTA GCA GTT CGT TTA GAA  954
 169 G   A   E   Y   A   V   L   P   E   L   A   V   R   L   E   183

 955 TAC CAA TGG CTA ACT CGC GTA GGT AAA TAC CGC CCT CAA GAT AAA  999
 184 Y   Q   W   L   T   R   V   G   K   Y   R   P   Q   D   K   198

1000 CCA AAT ACC GCA ATT AAC TAC AAC CCT TGG ATT GGT TGT ATC AAT 1044
 199 P   N   T   A   I   N   Y   N   P   W   I   G   C   I   N   213

1045 GCG GGT ATT TCT TAC CGT TTC GGT CAA GGC GAA GCA CCA GTT GTT 1089
 214 A   G   I   S   Y   R   F   G   Q   G   E   A   P   V   V   228

1090 GCA GCA CCT GAA ATG GTA AGC AAA ACT TTC AGC TTA AAT TCT GAT 1134
 229 A   A   P   E   M   V   S   K   T   F   S   L   N   S   D   243

1135 GTA ACT TTC GCA TTT GGT AAA GCA AAC TTA AAA CCT CAA GCA CAA 1179
 244 V   T   F   A   F   G   K   A   N   L   K   P   Q   A   Q   258

1180 GCT ACA TTA GAC AGC GTC TAT GGC GAA ATT TCA CAA GTT AAA AGT 1224
 259 A   T   L   D   S   V   Y   G   E   I   S   Q   V   K   S   273

1225 CGA AAA GTA GCT GTT GCT GGT TAC ACT AAC CGT ATT GGT TCT GAC 1269
 274 R   K   V   A   V   A   G   Y   T   N   R   I   G   S   D   288
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Figure 5 (cont.)

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1270 GCG TTC AAC GTA AAA CTT TCT CAA GAA CGT GCA GAT TCA GTA GCT  1314
 289 A   F   N   V   K   L   S   Q   E   R   A   D   S   V   A    303

1315 AAC TAC TTT GTT GCT AAA GGT GTT GCA GCA GAC GCA ATC TCA GCA  1359
 304 N   Y   F   V   A   K   G   V   A   A   D   A   I   S   A    318

1360 ACT GGT TAC GGT GAA GCA AAC CCA GTA ACT GGC GCA ACT TGT GAC  1404
 319 T   G   Y   G   E   A   N   P   V   T   G   A   T   C   D    333

1405 CAA GTT AAA GGT CGT AAA GCA CTT ATC GCT TGT CTT GCT CCA GAC  1449
 334 Q   V   K   G   R   K   A   L   I   A   C   L   A   P   D    348

1450 CGT CGT GTA GAA ATC GCA GTA AAC GGT ACT AAA TAA ttttagtcgttt 1497
 349 R   R   V   E   I   A   V   N   G   T   K   *                360

 1498 aacgaaagattaaatacaggaaaaggcttaaacttcggtttaggccttttgttttaaacg 1557

1558 aaactaaaaccaagcattttaatcaagttttaacttgtgataaaatgcttacctcgttta 1617

1618 tttataggaaacattatggaaaccttagacaaaatcaaaaaaagcaaattagtgaaaacc 1677

1678 ccattcttatttatatgaaaggttcgccaaaagtttccatcct                  1720
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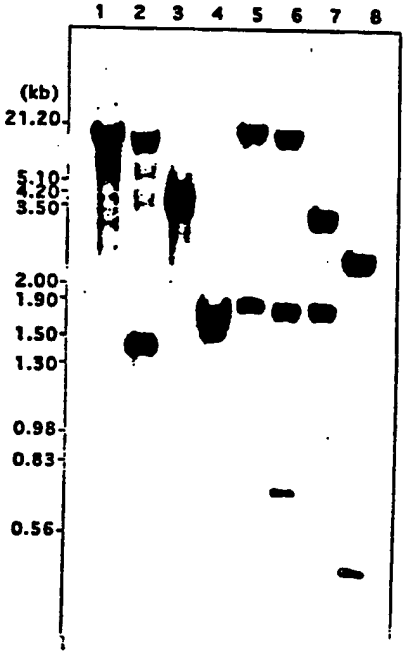
Figure 6

A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1.50
1.30
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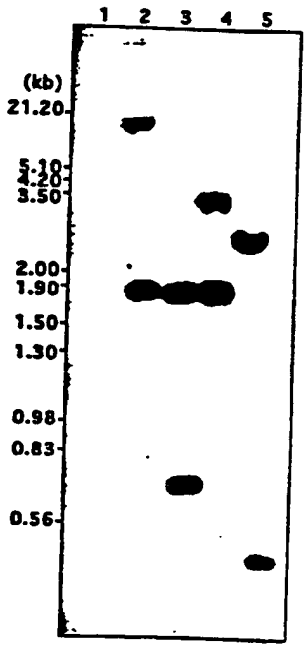
0.83
0.56

B


1 2 3 4 5
(kb)
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0.83
0.56

Figure 7

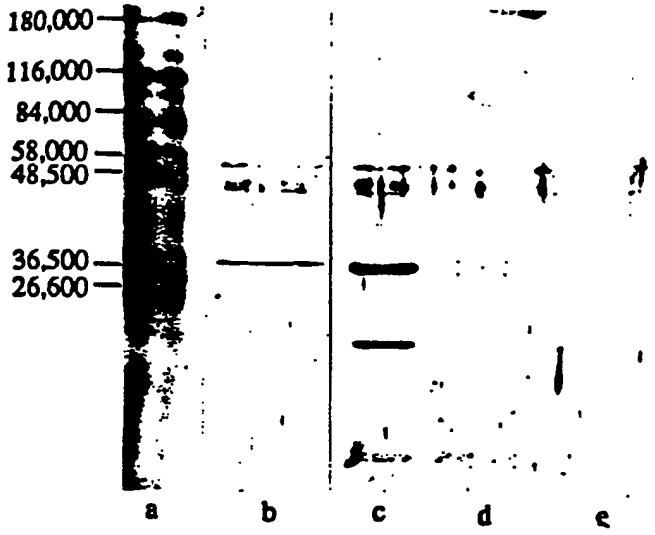


Figure 8

Figure 9

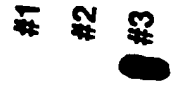
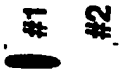


Figure 10



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/05477

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/02; C07K 15/12, 17/00; C12N 1/00, 1/20, 15/00
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/ 242.1, 256.1 ; 536/27; 435/ 69.1, 69.3, 71.1, 243, 252.3, 320.1; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Abstracts of The Fifth International Symposium RECENT ADVANCES IN OTITIS MEDIA, issued 20-24 May 1991, Bakaletz et al, "Presumptive identification of the NTHi Adhesion For Human Oropharyngeal And Chinchilla Middle Ear Epithelial Cells", abstract no. 132, see entire abstract.	9, 10, 25-27
X	The Journal of Infectious Diseases, Volume 165, Suppl. 1, issued June 1992, Bakaletz et al, "Cloning and Sequence Analysis of a Pilin-Like Gene from an Otitis Media Isolate of Nontypeable *Haemophilus influenzae*", pages S201-S203, see entire document.	11-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 22 AUGUST 1994	Date of mailing of the international search report SEP 02 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer H. F. Sidberry Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTER[redacted]IONAL SEARCH REPORT

International application No.
PCT/US94/05477

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Infection and Immunity, Volume 57, No. 10, issued October 1989, Bakaletz et al, "Immunological Responsiveness of Chinchillas to Outer Membrane and Isolated Fimbrial Proteins of Nontypeable *Haemophilus influenzae*", pages 3226-3229, see pages 3227 and 3226, 3rd paragraph.	1-8, 10, 25-29
X	Abstracts of the Fifteenth Midwinter Research Meeting, Association For Research in Otolaryngology, issued 2-6 February 1992, Bakaletz et al, "Protection Of Chinchillas Against Experimental Otitis Media Via Active Immunization with NTHi Strain #1128 Fimbrin", Abstract #219, see entire abstract.	1-8, 25-29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/05477

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/ 242.1, 256.1 ; 536/27; 435/ 69.1, 69.3, 71.1, 243, 252.3, 320.1; 530/350